

From the Division of Clinical Microbiology
DEPARTMENT OF MICROBIOLOGY, TUMOR AND CELL BIOLOGY
- MTC
Karolinska Institutet, Stockholm, Sweden

EXTENDED-SPECTRUM β -LACTAMASE-PRODUCING ENTEROBACTERIACEAE

EPIDEMIOLOGY AND DYNAMICS OF FECAL CARRIAGE

Emilia Titelman, MD



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ABSTRACT

ESBL-producing Enterobacteriaceae (EPE) has become a major cause of community acquired urinary tract infection (UTI), and fecal carriage of EPE is emerging worldwide. The aims of this thesis were to study the molecular epidemiology of ESBL-enzymes in Stockholm (**I**) to evaluate treatment alternatives to the carbapenems for EPE (**I-III**), and to study the duration of fecal carriage and identify factors associated with prolonged carriage (**IV**).

Paper I describes a consecutive collection of EPE (n=169). The distribution of ESBL-enzymes and clonal relatedness of the isolates was determined with PCR, DNA sequencing and pulsed-field gel electrophoresis (PFGE). Antimicrobial activity was evaluated using gradient test, broth microdilution and disk diffusion, and the susceptibility test methods were compared for parenteral β -lactams. We found that CTX-M-15 (75%) and CTX-M-14 (23%) were the dominating genotypes, that the collection was largely polyclonal and that 41% of *E. coli* belonged to the international clone sequence type (ST) 131. We concluded that there are several oral (mecillinam, nitrofurantoin, fosfomycin) and parenteral (piperacillin-tazobactam, tigecycline, temocillin) treatment alternatives for *E. coli* but few for *K. pneumoniae*. We also showed that susceptibility rates obtained with Etest and disk diffusion (DD) were not in agreement with the reference method broth microdilution for piperacillin-tazobactam (TZP). Etest and DD are therefore not reliable to detect resistance to TZP, with the breakpoints used at the time of the study.

In **paper II** the novel cephalosporin CXA-101 (later known as ceftolozane) in combination with tazobactam (CXA-201) was evaluated against the same collection of isolates as in paper I, and compared to other β -lactam/ β -lactamase inhibitors. MICs were determined with broth microdilution and susceptibility to CXA-201 was 88-98%, depending on the concentration of tazobactam and the tentative breakpoint used. All ACL-resistant and 94% of the TZP-resistant isolates were CXA-201 susceptible. We concluded that ceftolozane-tazobactam (CXA-201) is a potential future therapeutic option against EPE, especially for TZP-resistant isolates.

Paper III evaluates the clinical and bacteriological activity of pivmecillinam for patients treated for lower UTI caused by an EPE (n=8). The clinical cure (resolved UTI symptoms after completed treatment) was high (8/8) but bacteriological cure ($< 10^3$ CFU/ml at follow-up after 30 days) was low (2/8), although none of the patients with persisting bacteriuria relapsed within 6 months.

In **paper IV** we studied the duration and dynamics of ESBL-carriage. A cohort of patients (n=61) were followed with fecal samples and questionnaires about antimicrobial treatment and risk factors for EPE, 1, 3, 6 and 12 months after EPE infection. EPE strains were subjected to PFGE, PCR for phylogrouping, detection of CTX-M phylogroup, *pabB* (ST131) and virulence genes and PCR based replicon typing. Patient and strain related variables were compared for carriers and non-carriers at 12 months. We concluded that EPE carriage is common 12 months after infection (43%) and that persisting carriage may be associated with *E. coli* phylogroup B2 and CTX-M-9. The strain background frequently changes throughout the carriage and negative samples do not imply eliminated carriage.

This knowledge will hopefully contribute to providing better medical care of patients with infection caused by EPE. It may also prove important for defining patients in require of prolonged isolation in single rooms or cohorts. Thereby the spread of EPE in hospitals and long term care facilities can be limited.

LIST OF PUBLICATIONS

- I. Titelman E, Iversen A, Kahlmeter and Giske CG. **Antimicrobial susceptibility to parenteral and oral agents in a largely polyclonal collection of CTX-M-14 and CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae*.** APMIS. 2011; 119: 853-863
- II. Titelman E, Karlsson I, Ge Y and Giske CG. **In vitro activity of CXA-101 plus tazobactam (CXA-201) against CTX-M-14 and 15-producing *E. coli* and *K. pneumoniae*.** Diagnostic Microbiology and Infectious Diseases. 2011; 70: 137-141
- III. Titelman E, Iversen A, Kalin M and Giske CG. **Efficacy of pivmecillinam for lower urinary tract infection caused by Extended-spectrum β -lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*.** Microbial Drug Resistance. 2012; 18: 189-192
- IV. Titelman E, Chowdhury MH, Iversen A, Kais M, Kalin M and Giske CG. **Fecal carriage of Extended-spectrum β -lactamase-producing *Enterobacteriaceae* is common twelve months after infection and is related to strain factors.** Submitted

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LIST OF ABBREVIATIONS

AmpC	Ampicillinase C
<i>bla</i>	Gene encoding β -lactamase
BSI	Bloodstream infection
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-resistant Enterobacteriaceae
CTX-M	Cefotaximase Munich
EARS-Net	European Antimicrobial Resistance Surveillance Network
ESBL	Extended-spectrum β -lactamases
ESBL _A	”Classical” ESBL (SHV-, TEM-variants and CTX-M)
ESBL _M	Miscellaneous ESBL (plasmid-mediated AmpC)
ESBL _{CARBA}	Carbapenemases (KPC, NDM, VIM and OXA-variants)
EPE	ESBL-producing Enterobacteriaceae
EUCAST	European Committee of Antimicrobial Susceptibility Testing
<i>fim</i>	Genes encoding Type1-fimbriae (surface structure of bacteria involved in adhesion)
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
MDR	Multi-drug Resistance
MIC	Minimum Inhibitory Concentration
NDM	New Delhi Metallo- β -lactamase
OXA	Oxacillinase-type β -lactamase
<i>pap</i>	Pili associated with pyelonephritis. P-fimbriae (surface structure of bacteria involved in adhesion)
PCR	Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
SHV	Sulphydryl variable (a type of β -lactamase)
ST131	Sequence type 131, an international clone of <i>E. coli</i>
TEM	Temoneira (a type of β -lactamase named after a patient)
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo- β -lactamase

1 INTRODUCTION

The discovery of antimicrobial agents was one of the greatest medical breakthroughs of the 20th century that revolutionized the treatment of bacterial infections.

Unfortunately the widespread use and misuse of antibiotics have resulted in a gradual emergence of antimicrobial-resistant bacteria, and multiresistance in various species has become a major threat. In the 21st century there has been an alarming trend with increasing multiresistance among bacteria belonging to the Enterobacteriaceae. The Enterobacteriaceae is a large family of Gram-negative, rod-shaped, non-fermenting facultative anaerobe bacteria. Most Enterobacteriaceae are members of the normal intestinal flora of humans and animals, others are found in water or soil. However, the group also includes common human pathogens, of which *Escherichia coli* and *Klebsiella pneumoniae* are considered the most important (Mandell 2000, Schaechter 1993). *E. coli* and *K. pneumoniae* cause various types of infections, including urinary tract infection (UTI), bloodstream infection (BSI) and severe hospital-acquired infections. UTI is the most common type of infection, with *E. coli* accounting for 70–95% of the community onset and approximately 50% of the nosocomial cases. Around 20% of adult women suffer from at least one UTI in their lifetime, and recurrent or relapsing UTIs are problematic in many individuals (Foxman 2010). Hence, widespread multiresistance among *E. coli* will have major implications.

Infections caused by *E. coli* and *K. pneumoniae* are normally treated with β -lactam antibiotics. The β -lactams is a broad class of antimicrobials that contain a four-atom ring (β -lactam ring) in their molecular structure. They work by inhibiting cell wall synthesis in the bacterial organism. The group includes the penicillins, cephalosporins e.g. cefotaxime, ceftriaxone and ceftazidime, monobactams and carbapenems. Resistance among *E. coli* and *K. pneumoniae* is most often due to inactivation of these antibiotics by enzymes through hydrolysis of the β -lactam ring (Fig. 1), so called extended-spectrum β -lactamases (ESBLs). ESBLs are able to break down penicillins, cephalosporins, monobactams and sometimes carbapenems (Giske et al. 2009).

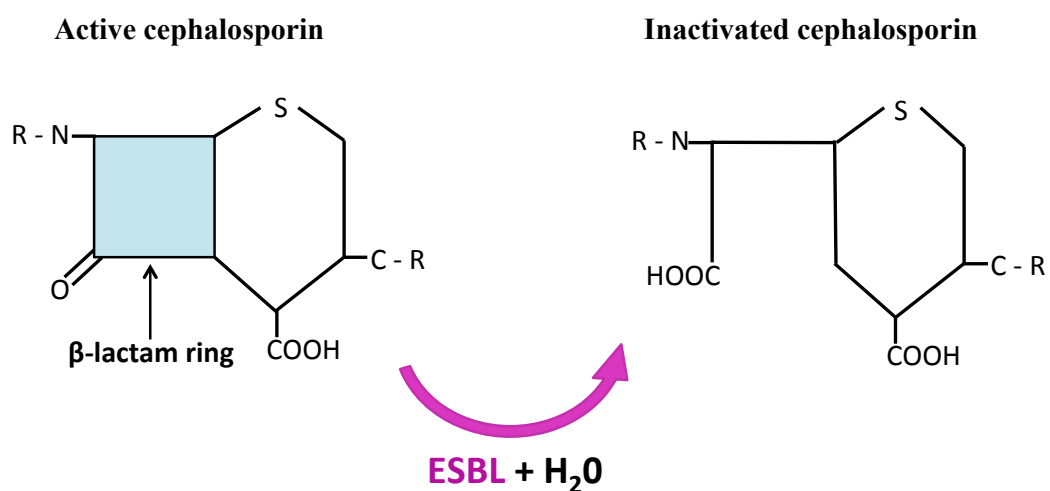


Figure 1. Inactivation of a cephalosporin through hydrolysis of the β -lactam ring by an ESBL

Infections caused by ESBL-producing Enterobacteriaceae (EPE) are not more severe than similar infections caused by non-ESBL-producing strains. However, because the ESBL inactivates the most commonly used antimicrobial agents, empirical treatment is often ineffective and adequate treatment delayed. Therefore, infections with EPE are associated with increased mortality, length of hospital stay and health care costs (Giske et al. 2008, Rottier et al. 2012).

ESBL genes are transferable between bacteria by horizontal gene transfer through plasmids. A plasmid is a double-stranded, extra-chromosomal mobile genetic element that replicate independently of the chromosome. Plasmids carry genes non-essential for bacterial survival although they provide benefits to the bacterium by encoding genes for virulence, environmental adaptability and persistence, metabolic functions and resistance to heavy metals and antibiotics.

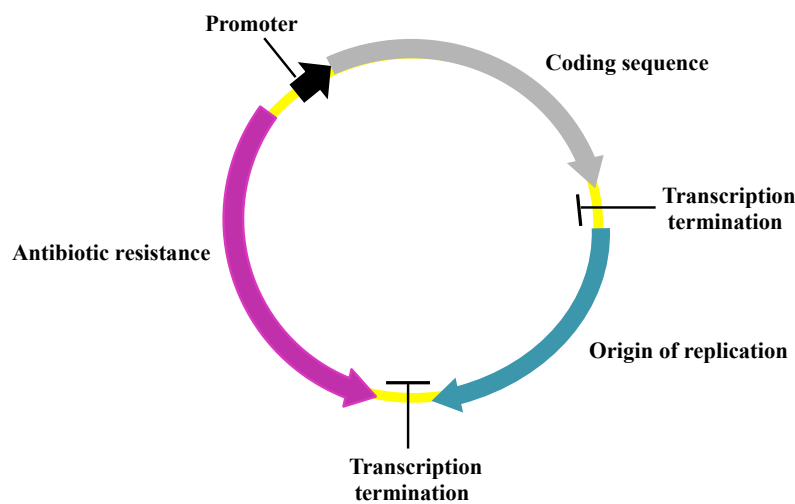


Figure 2. Schematic structure of a plasmid

Plasmids replicate independently of the chromosome and partition themselves between daughter cells after cell division. Some plasmids are also able to transfer themselves to other bacteria through bacterial conjugation. The principles of conjugation are shown in Fig. 3.

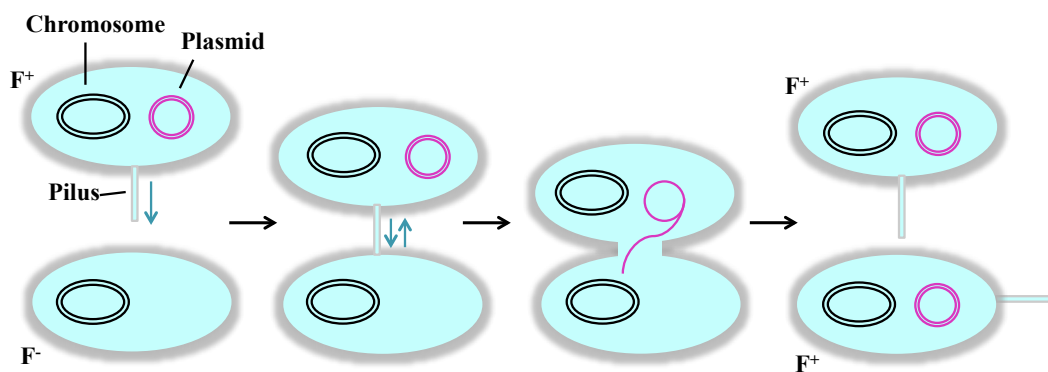


Figure 3. Principles of bacterial conjugation

Plasmids vary in size and carry between one and over one thousand genes. Larger plasmids are present in smaller numbers (1-2) and small plasmids may be found in high copy numbers (~40). Plasmids that are closely related and share common replication control systems cannot be propagated stably in the same cell line; hence, they are incompatible and classified into the same incompatibility group (Inc) (Datta and Hedges 1971). There are >30 Inc groups described, and major groups occurring in Enterobacteriaceae are HI2, HI1, I1- γ , X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O, FII, FIII, FIV. ESBL-genes are often found on IncF, I and N plasmids (Carattoli 2013). IncF plasmids are large low-copy-number plasmids that often carry more than one replicon. These plasmids often also harbour other resistance genes. Therefore, ESBL-producing bacteria are not only resistant to β -lactams but frequently also to other groups of antibiotics including aminoglycosides, quinolones and trimethoprim-sulfamethoxazole (Paterson and Bonomo 2005, Pitout 2012).

There is a large number of ESBL-variants, and new enzymes are frequently being detected. In 2009 the definition of ESBL was broadened and now includes three classes of β -lactamases (ESBL_A, ESBL_M, and ESBL_{CARBA}) (Giske et al. 2009) shown in Table 1. However, in the literature, ESBL most often refers to the “classical” and most common ESBLs, ESBL_A, which includes the dominating CTX-M-enzymes. ESBL_M shares phenotype with ESBL_A but is less frequent and not so well studied. ESBL_{CARBA} is resistant also to the carbapenems and is therefore an even greater challenge for health care, although still very rare in Sweden. In this thesis ESBL refers to ESBL_A.

Table 1. Classification of ESBL enzymes

Class of ESBL	Ambler class	Enzymes	Inhibited by
ESBL _A	A	CTX-M, TEM/SHV-ESBL	Clavulanic acid
ESBL _M	C	Plasmid AmpC	Cloxacillin
ESBL _{CARBA}	A	KPC	Boronic acid
	B	Metallo- β -lactamases (VIM, NDM, IMP)	EDTA
	D	OXA-48	No inhibitor*

*Inhibited by the new β -lactamase-inhibitor avibactam, no commercial assays available yet. Resistant to temocillin (www.nordicast.org).

The past decade the CTX-M-enzymes have emerged globally and ESBL has gone from being a rare problem associated with *K. pneumoniae* hospital-acquired infections, to a major cause of community-acquired *E. coli* UTI. Asymptomatic fecal carriage of EPE among healthy individuals has become endemic in many countries and is spreading rapidly throughout the world through food and water resources, with the help from travellers. When this project was initiated in 2006, an increase in EPE had been noted also in Sweden. Data on their local molecular epidemiology was non-existing and needed to be addressed. Today EPE is a clinical problem that physicians deal with on an everyday basis. Therapeutic options for infections caused by EPE are few, and primary care patients with UTI are frequently admitted for intravenous carbapenem treatment. The broad-spectrum carbapenems are considered the drugs of choice, but are not suited for treatment of non-severe infections. Apart from being inconvenient and

expensive, the increasing use of the carbapenems contributes to the selection of resistant strains in the gut (Falagas 2012), and has led to an alarming rise in carbapenem resistance in many countries (Nordmann et al. 2009). The use of carbapenems must be reduced, but what are the alternatives? Data on the activity of other agents against EPE is limited, and old as well as novel agents, need to be evaluated against ESBL-producing isolates (Talbot et al. 2006).

Along with the increasing detection of EPE, the prevalence and risk factors for fecal carriage have been described. However, little is known about the time course and dynamics of stool colonization of EPE. The lack of knowledge constitutes a problem not only from an infection control perspective, but also poses a problem to clinicians having to inform patients about the finding of EPE in clinical or screening samples. How long does one carry the bacteria? Can they be eliminated? Which patients are at risk for prolonged carriage? How long do we need to consider these bacteria as potential pathogens and what patients require cohorting and isolation upon readmission to prevent the spread to others within the hospital? These are important and frequently asked questions that cannot be answered without increased knowledge on the duration of fecal carriage of ESBL.

The aims of the research presented in papers **I-IV** were to study the epidemiology of ESBLs in Stockholm (**I**), to evaluate existing and new treatment alternatives to the carbapenems (**I-III**), and to study the duration and dynamics of fecal carriage of EPE (**IV**). This was carried out through molecular investigations of a consecutive collection of EPE from Stockholm (**I, II**), and by following a cohort of patients after clinical infection caused by EPE (**III, IV**).

In the following sections I present and discuss findings from paper **I-IV** while placing them in a wider context. I begin by introducing the most common type of ESBL, the CTX-M-enzymes, and describe their clinical dissemination and molecular epidemiology, globally and in Stockholm (**I**). I then proceed to discussing treatment alternatives to the carbapenems, while highlighting some of the findings from paper **I-III**. I end with a section on fecal carriage, with special emphasis on duration of carriage and host and pathogen factors possibly affecting it (**IV**). Details on materials, methods, results and discussion can be found in the papers and will not be repeated unless it is relevant for the understanding. ESBLs constitute an extensive field of research, and my intention is not to present a voluminous review, but to give an understanding of the work presented in the papers.

2 CLINICAL DISSEMINATION AND MOLECULAR EPIDEMIOLOGY OF EPE

2.1 THE CTX-M-ENZYMES

The first ESBL was described in 1983 (Knothe et al. 1983). Until the end of the 1990s the majority of the ESBL-enzymes reported were of the TEM- (Temoneira, named after a patient) or SHV- (Sulf-hydryl variable) types. These enzymes had evolved from the previously known penicillinases TEM-1 and 2 and SHV-1 by point mutations, and were mostly produced by nosocomial bacteria, especially *Klebsiella spp*, and found in patients with severe hospital-acquired infections. Risk factors for acquisition were long term hospital stays, especially in the intensive care unit, intubation, catheterization and previous exposure to antimicrobials. The spread was clonal, and many cases were related to outbreaks in hospital wards (Paterson and Bonomo 2005).

In 1986 a new type of ESBL was found in the feces of a Japanese dog. This enzyme had an elevated activity against cefotaxime and was therefore named CTX-M (CefoTaXimase, first isolated in Munich) after being isolated in Germany in 1989 (Bauernfeind et al. 1990). Originally, the CTX-M genes were picked up from the chromosomes of the environmental bacteria *Kluyvera spp* by mobile genetic elements, and had continued to spread to other species through bacterial conjugation of plasmids. Since 2000 the CTX-M enzymes have emerged globally (Canton and Coque 2006), and in a multinational study from 2009, CTX-M enzymes accounted for 65% of all β -lactamases (Ben-Ami et al. 2009). While SHV and TEM are mostly limited to nosocomial outbreaks, the CTX-M enzymes are widely spread in the community. CTX-M-producing Enterobacteriaceae has become an important cause of community onset UTI (Pitout and Laupland 2008).

There is a large number of CTX-M variants, and novel enzymes are frequently being described, reflecting their rapid spread and evolution. There are currently >100 different CTX-M-enzymes, divided into six groups (1, 2, 8, 9, 25 and 45), based on their amino-acid sequences (Bonnet 2004, Rossolini et al. 2008). CTX-M-15, which belongs to the CTX-M-1-group, is the most widely spread enzyme internationally (Oteo et al. 2010) and in Sweden (I). CTX-M-15 is not only the most common ESBL-enzyme, it is also associated with the most multi-resistant phenotype (Pitout 2012, I). The highly resistant profile often described with *E. coli* producing CTX-M-15 may be explained by the association of CTX-M-15 with the co-production of other β -lactamases (TEM-1, OXA-1) and the aminoglycoside-modifying enzyme Aac(6')-Ib-CR. Aac(6')-Ib-CR not only accounts for aminoglycoside-resistance but can also cause flouoroquinolone-resistance through acetylation. The production of CTX-M-15, TEM-1, OXA-1 and Aac(6')-Ib-CR has been linked to epidemic IncFII plasmids (Peirano and Pitout 2010).

2.1.1 CTX-M-15 and the international *E. coli* clone ST131

The high spreading capacity of CTX-M-15- producing *E. coli* could be explained by

the horizontal gene transfer through plasmids harbouring *bla*_{CTX-M-15}, or by the spread of an epidemic clone with selective advantages. The literature supports that the international dissemination of CTX-M-15 is at least in part due to the rapid spread of the international *E. coli* clone sequence type 131 (ST131) identified through multilocus sequence typing (MLST) (Oteo et al. 2010). ST 131 is a gut colonizer and uropathogen, belonging to the most virulent *E. coli* phylogroup B2 (serotype 025b-H4). It has acquired the IncFII-plasmid containing the CTX-M-15, TEM-1, OXA-1, *aac*(6')-Ib-cr complex, and by this gained the selective advantage of multiple antimicrobial resistance genes in addition to its enhanced virulence factors (Pitout). This unfortunate combination has led to the spread of CTX-M-15 between hospitals, long-term care facilities and, not the least, in the community. ST131 is believed to have spread through food and contaminated water resources and ESBL, including CTX-M-15 and other CTX-M-types belonging to ST131, have been found in farm animals, food, sewage, rivers and lakes (Mesa et al. 2006). Several studies indicate that travellers help transporting the bacteria between countries and continents (Tangden et al. 2010, van der Bij and Pitout 2012). Fecal carriage of ESBL has also been reported to be widespread among wild populations including migrating birds (Guenther et al. 2012). Wildlife may serve as an environmental reservoir for ESBL and humans may be re-infected through contact with the animals' feces. Bird migration could therefore contribute to the global dissemination of ESBL, in similar ways that have been described for human travel.

2.2 GLOBAL DISSEMINATION OF CTX-M-ENZYMES AND ST131

The proportion of ESBL-producing *E. coli* and *K. pneumoniae* varies geographically (3 to 80%). The highest prevalences are reported from Asia, Latin America and some European countries (Oteo et al. 2010). In the Asia-Pacific region and in Latin America 40% and 30% respectively of *E. coli* and *Klebsiella spp.* from patients with intra-abdominal infections have been reported ESBL-positive. In India as many as 79% of clinical isolates of *E. coli* have been found to be ESBL-producing. In China and Thailand the published numbers are around 50% (Hawser et al. 2009). In Europe the highest rates are found in the southern and eastern parts (10 to 25%), as displayed in Fig. 4, which shows the percentage of invasive *E. coli* isolates with resistance to third-generation cephalosporins by country in 2011. However, it should be noted that some countries do not have the routine to report all isolates, which may lead to an overestimate of the proportion of ESBL-producing isolates.

Specific CTX-M genotypes are associated with different geographical regions. CTX-M-15 is the most widely spread genotype followed by CTX-M-14 and CTX-M-3. CTX-M-14 and CTX-M-3 are common in China, Japan, South East Asia and North America. CTX-M-2 dominates in South America (Hawkey and Jones 2009). In Europe, the majority of the enzymes belong to the CTX-M-1-group (CTX-M-3 and -15) and the CTX-M-9-group (CTX-M-9 and -14) (Oteo et al. 2010). In India, with its large population and extremely high frequency of EPE, no other genotype than CTX-M-15 has been described. Also in regions with a tradition of other dominating enzymes, like China and the USA, CTX-M-15 is catching up and replacing the other enzymes (Hawkey and Jones 2009). In reports from the USA as well as from Canada, Norway and Spain, the majority of the CTX-M-15 isolates have been related to ST131 (Nicolas-Chanoine et al. 2008, Peirano and Pitout 2010).

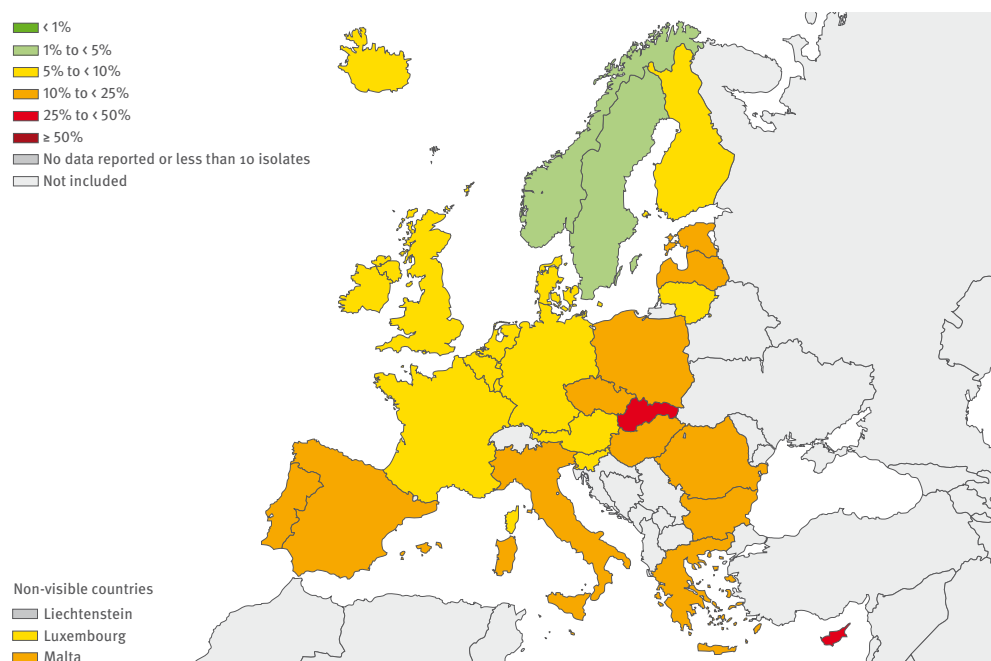


Figure 4. Percentage (%) of invasive isolates with resistance to third-generation cephalosporins by country, EU/EEA countries, 2011 (EARS-Net database 2011).

2.2.1 CTX-M-enzymes and ST131 in Sweden

When this work was initiated in 2006, there was no data on the prevalence of ESBLs in Sweden. Mandatory laboratory reporting was introduced in 2007, and has shown that EPE is by far the most common and fastest increasing type of multiresistance in the country, with 7,225 reported cases during 2012 and 1,880 cases in Stockholm (Fig. 5).

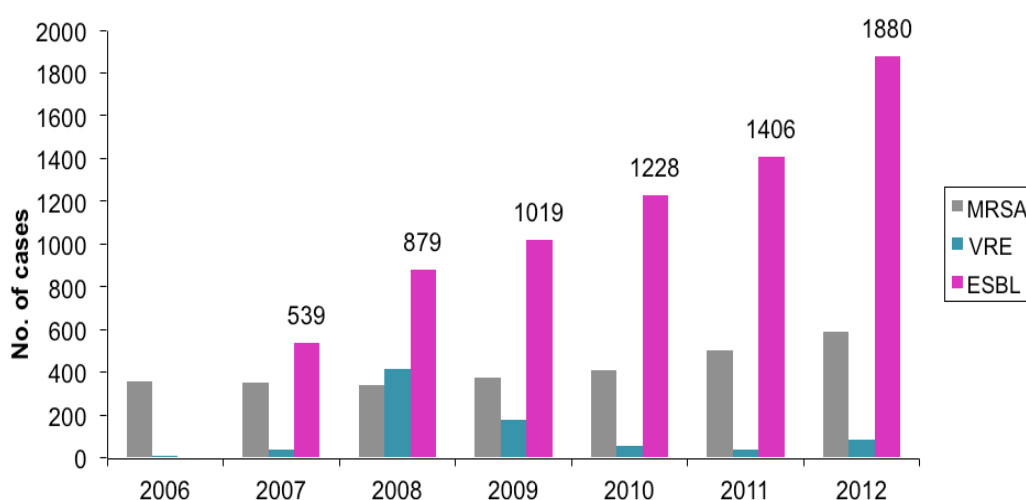


Figure 5. Notifiable resistance in Stockholm 2006-2012 (The Swedish Institute for Communicable Disease Control (www.smittskyddsinstitutet.se)).

The proportion of cephalosporin-resistant isolates in blood is now (2011) around 3% in the country (Fig. 4). According to the SWEDRES-report from the same year, which presents data on a national level from the Swedish surveillance of antibiotic resistance

programme, the most commonly reported species with ESBL was *E. coli*, accounting for 87% of all cases, followed by *K. pneumoniae* with 7% and *Proteus mirabilis* (0.5%). In 63% of the cases the EPE was detected in a urine sample, and 22% in fecal samples or rectal swabs. In additional genotypic analyses of 508 cefadroxile-resistant urine isolates collected from all clinical microbiology laboratories in Sweden during one month in 2011, 82% were ESBL-producers. Of these, 93.3% had ESBL_A, 6.3% ESBL_M, and 0.5% both ESBL_A and ESBL_M. Among the ESBL_A, CTX-M belonging to group 1 was the most prevalent enzyme (73.5%), followed by CTX-M group 9 (24.5%), and other type(s) (3%) (SWEDRES 2011).

In paper I we characterized a consecutive collection of all clinical ESBL-producing isolates referred to the Karolinska University hospital in 2005 (n=169). The Karolinska University hospital covers the majority of the population of Stockholm. The prevalence of ESBL was 0.7% among *E. coli* and *K. pneumoniae* in 2005 (I). We found that, as described above in more recent Swedish collections as well as in many other countries, CTX-M-15 was the dominating genotype followed by CTX-M-14 (I) (Fig. 6). The results were obtained with specific PCRs and subsequent DNA-sequencing, and only CTX-M-negative isolates were run with primers targeting TEM and SHV. Hence, it is possible that some of the CTX-M isolates harboured additional TEM and/or SHV-genes (although not the opposite).

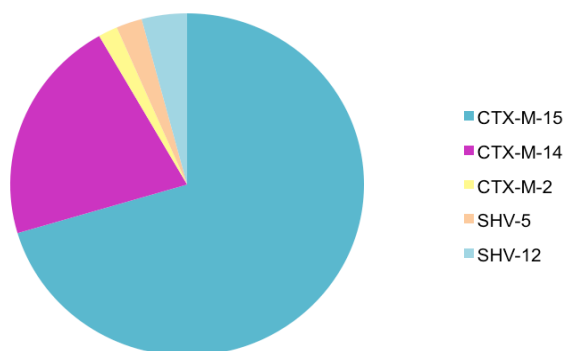


Figure 6. Distribution of ESBL genotypes in Stockholm 2005 (modified from paper I).

Apart from national data from SWEDRES, several other studies are supporting that CTX-M-15 is the most prevalent genotype in Sweden (Fang et al. 2004 and 2008, Cars et al. 2007, Lytsy et al. 2008, Onnberg et al. 2011, Tärnberg et al. 2013, Helldal et al. 2013.). The largest study, by Tärnberg et al, showed a similar distribution of CTX-M enzymes in Linköping 2002-2007 as in our study (I) with 67% of 198 *E. coli* ESBL-isolates belonging to group 1 and 28% to group 9. However, paper I was the first Swedish study of ESBL-genotypes based on a consecutive collection from a whole year in a non-outbreak situation. Epidemiological typing using pulsed-field gel electrophoresis (PFGE) showed that the collection was largely polyclonal, indicating that the spread in Stockholm occurred through horizontal gene-transfer by plasmid conjugation. Further, we found that the rate of *E. coli* isolates belonging to ST131 was 41 % (I). Fig. 7 shows the clonal relatedness of the isolates and whether they were ST131-positive. In paper IV, which was a smaller study (n=61, *E. coli* n= 56), 29% of the isolates belonged to ST131. Seven of the 14 patients infected with an *E. coli* belonging to ST131 had travelled abroad within six months before their infection, suggesting that they might have picked up the EPE during the travel.

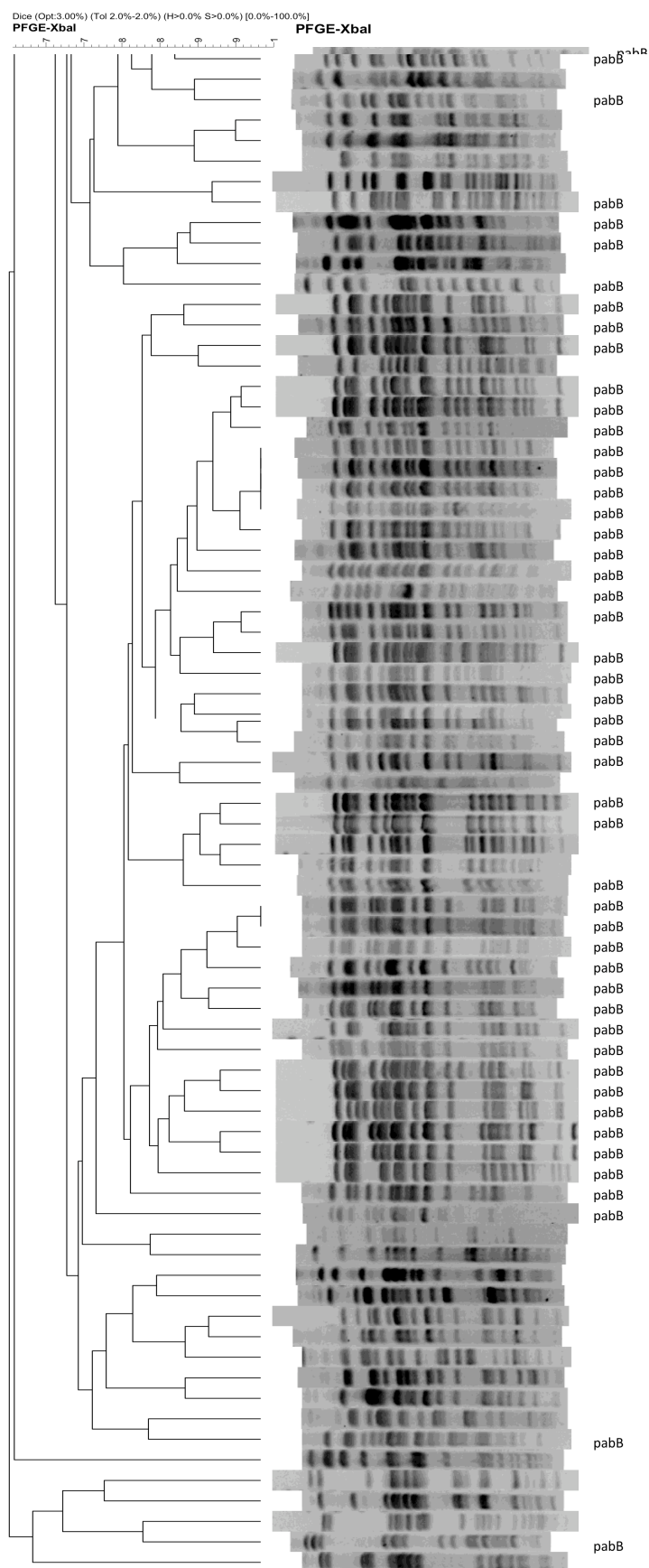


Figure 7. Clonal relatedness of clinical isolates of ESBL-producing *E. coli* referred to the Karolinska University Hospital in Stockholm in 2005. Isolates marked *pabB* belong to ST131 (paper I). Continued from previous page.

2.3 ESBL_{CARBA}

ESBLs that also inactivate carbapenems, so called carbapenemases or ESBL_{CARBA}, pose an even greater threat than other ESBLs because they limit the treatment options further. As for other ESBLs ESBL_{CARBA} is mainly produced by *E. coli* and *K. pneumoniae*. Most carbapenemases of clinical importance belong to KPC (*K. pneumoniae* carbapenemase), NDM (New Delhi metallo- β -lactamase, VIM (Verona integron-encoded β -lactamase) or OXA-48 (oxacillinase). Their prevalence varies geographically. NDM is the most common enzyme in South-East Asia and the Middle East whereas KPC is dominating in Europe where OXA-48 is also increasingly reported. In Europe, the highest numbers of carbapenemase producing Enterobacteriaceae (CPE) are seen in Greece, Italy, Turkey and Israel followed by Spain, France and the UK. In the Nordic countries the rates are still very low (Canton et al. 2012). However, as displayed in Fig. 8, ESBL_{CARBA} is increasing also in Sweden.

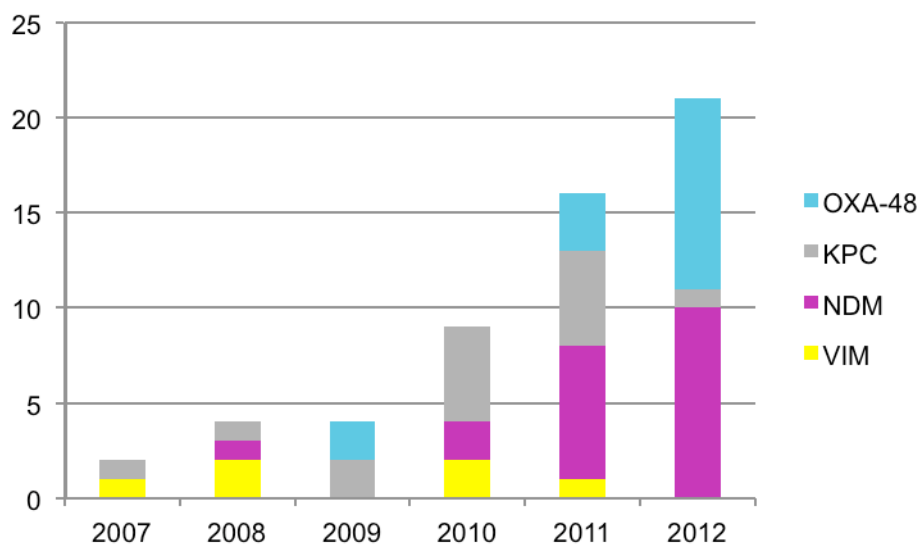


Figure 8. Numbers and types of ESBL_{CARBA} in Enterobacteriaceae in Sweden 2007-2012 (SWEDRES 2012).

Most cases have been associated with contact with health care in endemic countries such as Greece (KPC and VIM) and India (NDM). However, as for the CTX-M-enzymes, spread of ESBL_{CARBA} is becoming more frequently reported in the community. Thus, an increasing acquisition among travelers is to be expected in the future. Rapid identification of colonized or infected patients and screening of carriers is important to prevent an endemic scenario similar to that of the CTX-M-enzymes. To facilitate this work, reporting of ESBL_{CARBA} became mandatory not only for laboratories but also for clinicians in Sweden in 2012.

3 TREATMENT ALTERNATIVES FOR EPE-INFECTIONS

3.1 THE DRUGS OF CHOICE: THE CARBAPENEMS

ESBL inactivates most β -lactam antibiotics and co-resistance to quinolones, trimethoprim-sulfamethoxazole and aminoglycosides is frequently observed. When treating ESBL-infections, one must take into account not only the agents' activity in vitro, but also the clinical activity against EPE specifically. The impact on the normal fecal flora and the association with selection of ESBL-producing strains should also be considered. Most EPE are susceptible to the carbapenems (Paterson and Bonomo 2005, Pitout and Laupland 2008, **I**). Meropenem, imipenem and doripenem have all shown excellent clinical effect against severe ESBL-infections (Chaubey et al. 2010), and ertapenem against UTI (Bazaz et al. 2010). Therefore, the carbapenems are considered the drugs of choice against ESBL-infection. However, EPE may develop resistance to the carbapenems due to loss of porins which reduces the permeability for the antibiotic (Doumith et al. 2009). In general EPE-isolates display lower MICs for meropenem and doripenem than imipenem and ertapenem. Resistance to ertapenem is more common than resistance to other carbapenems (Woodford et al. 2007). Meropenem and doripenem are therefore recommended for empirical treatment of severe EPE-infection. However, there are several reasons to limit the use of the carbapenems: First, the use contributes to the selection of resistant strains in the gut (Falagas et al. 2013). Second, increasing rates of ESBL_{CARBA} and other types of carbapenem resistance are being reported (Nordmann et al. 2009) and we need to "save it" for the most severe cases. Third, carbapenems are only administrated intravenously and not suitable for the treatment of non-severe infections. Thus, we need treatment alternatives, both for parenteral and oral use.

There are several existing agents that have demonstrated high activity against EPE in vitro, although we lack data to support their clinical utility. Susceptibility data for EPE varies geographically and may also depend on the type of ESBL. In addition, the outcome of the susceptibility testing may be affected by the methods used in the laboratory, at least for certain agents (Pitout et al. 2008). In paper **I**, one of the specific aims was to evaluate possible treatment alternatives to the carbapenems in our setting. The susceptibility to a large number of oral and parenteral agents were tested against a consecutive collection of 169 EPE isolates collected in Stockholm during 2005. At the time, data on resistance-profiles for the various ESBL-genotypes were limited. However, there were indications that the CTX-M-15 had the most multi-resistant phenotype, which has later been confirmed (Pitout 2010, **I**). To learn more about this, susceptibility-rates were related to ESBL genotype. As expected, high resistance-levels were found for ciprofloxacin, trimethoprim and gentamicin among *E. coli* (28, 29 and 53%). For *K. pneumoniae* we found high resistance to all antibiotics, although for *E. coli* we found several possible treatment alternatives both among parenteral and oral agents. Table 2 shows the agents for which we found the highest susceptibility rates for *E. coli*. The agents were compared to the carbapenems, here represented by ertapenem. Each of these agents is being discussed below.

Table 2. Antimicrobial susceptibility and ESBL genotypes, *E. coli* (paper I).

Genotype (No.)	Antimicrobial susceptibility*						
	% susceptible isolates						
	ERT	TGC	TZP	TEM	FOS	NIT	MEC
<i>bla</i> _{CTX-M-14} (30)	100	100	100	93	97	94	90
<i>bla</i> _{CTX-M-15} (108)	99	100	87	70	98	94	89
<i>bla</i> _{CTX-M-2} (2)	100	100	100	100	0	100	100
<i>bla</i> _{SHV-5} (2)	100	100	100	50	100	100	0
<i>bla</i> _{SHV-12} (7)	86	100	100	43	86	86	57

*ERT=ertapenem; TGC=tigecycline; TZP=piperacillin/tazobactam; TEM=temocillin; FOS=fosfomicin; NIT=nitrofurantoin; MEC=mecillinam

3.2 β -LACTAM/ β -LACTAMASE-INHIBITOR COMBINATIONS

ESBLs can be inhibited in vitro by β -lactamase-inhibitors such as clavulanic acid and tazobactam, although their clinical activity have been questioned. Previously, ESBL-producing isolates were reported resistant to all β -lactams and β -lactam/ β -lactamase-inhibitor combinations (β L/ β LI). However, the Clinical Laboratory Standards Institute (CLSI) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) have lowered the breakpoints, and now recommend reporting susceptibility results for β -lactam antibiotics as found, regardless of the presence of an extended-spectrum β -lactamase. The impact of these recommendations on susceptibility rates among EPE has not been fully studied. However, β L/ β LI have emerged as interesting alternatives to the carbapenems because of their high activity in vitro and low association with development of ESBL-infections (Vardakas et al. 2012). Although comparative trials are limited, a recent review and meta-analysis by Vardakas et al, did not show inferiority to the carbapenems regarding mortality for empirical or definite treatment with β L/ β LI against BSI (Vardakas et al. 2012). A similar post-hoc analysis by Rodriguez-Bano et al. showed that amoxicillin/clavulanic acid and piperacillin/tazobactam were not associated with increased mortality compared to the carbapenems for the definite therapy of ESBL-*E. coli* BSI with origin in the urinary and biliary tract and susceptibility in vitro (Rodriguez-Bano et al. 2012).

3.2.1 Piperacillin/tazobactam

Apart from the studies of β L/ β LI against BSI mentioned above, current literature also supports the clinical action of piperacillin/tazobactam (TZP) for susceptible ESBL-producing isolates, at least in upper urinary tract infections (UTI) and pneumonia (Thomson and Moland 2001, Gavin et al. 2006, Rodriguez-Bano et al. 2006, Peterson 2008). With the increasing frequency of ESBL calling for a reduction in the use of the cephalosporins, the use of TZP has increased substantially in Swedish hospitals the last few years. In paper I we found high susceptibility rates (87%) to TZP in accordance with several previous reports, although the lowest activity was seen among CTX-M-15 isolates. However, these numbers were determined with

gradient MIC-testing (Etest), a method that had not been evaluated specifically for ESBL-isolates. Failure to detect resistance to TZP had previously been reported using automated systems (Pitout 2008). Therefore, we aimed to evaluate the accuracy of the two commonly used diffusion-based susceptibility testing methods, gradient MIC-testing (Etest) and disk diffusion. When determining MICs for TZP for the same isolates using Broth microdilution (BMD), which is considered the reference method, the susceptibility rate was only 58%. Thus, gradient MIC-testing was found to be unable to detect resistance to TZP in 56/169 cases. The discrepancies between Etest and broth microdilution are displayed in Fig. 9.

MIC Etest (mg/L)	MIC Broth microdilution (mg/L)							
	0.25	0.5	1	2	4	8	16	>32
0.25								
0.5								
1								3
2								6
4							10	11
8							13	13
16						1		
>32								

Figure 9. Discrepancies between Etest and Broth microdilution for piperacillin/tazobactam (modified from paper I).

Similar discrepancies were found when comparing susceptibility obtained with Disk diffusion and Broth microdilution (Fig. 10).

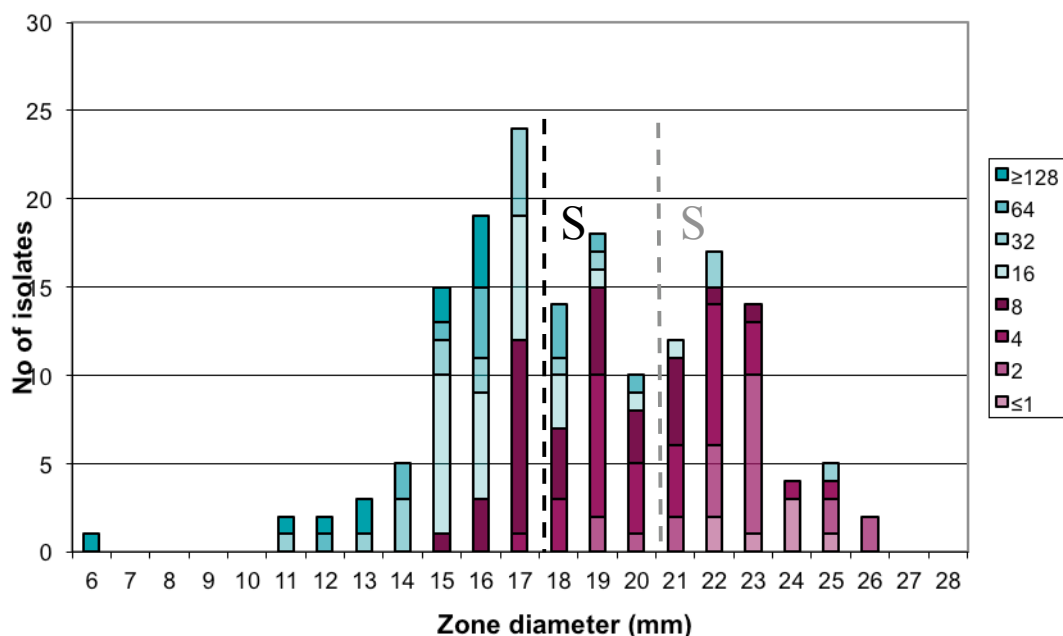


Fig 10. Broth microdilution vs Disk diffusion for piperacillin/tazobactam: discrepancies between MICs and zone diameters. The individual bars are coloured with the MICs of isolates having the various zone diameters. Several major errors occurred. Increasing the susceptibility breakpoint from 18 mm to 21 mm would, however, result in no major errors (modified from paper I).

When applying the old susceptibility breakpoint for disk diffusion (≥ 18 mm), a number of isolates were classified as susceptible although they were actually resistant (Fig. 10). Since this phenomenon was not known before our publication (I), it is possible that previously described treatment failures with TZP against EPE could have been due to non-susceptible isolates. Thus, the clinical activity against susceptible isolates might be better than has been reported. On the other hand, susceptibility rates may actually be lower than previously described. These data shows the importance of calibrating the breakpoints against the reference method, and have resulted in a revision of the EUCAST disk diffusion breakpoint for TZP which is now ≥ 20 mm.

In general, resistance to TZP is increasing among Gram-negative pathogens. This has been associated with more pathogens expressing multiple β -lactamases simultaneously. Among these β -lactamases there has been an accumulation of mutations that decrease their susceptibility to inhibition by tazobactam (Sirot et al. 1997, Shlaes 2013). Thus TZP is not the ultimate solution to the ESBL-problem, and more potent β L/ β LI are being evaluated.

3.2.2 Cephalosporin/ β -lactamase-inhibitor combinations

Cephalosporins have traditionally been considered non-effective against EPE-infection even with susceptibility in vitro (Paterson and Bonomo 2005). This problem has been shown to be related to the previously used higher cephalosporin breakpoints (Pitout 2010). In contrast to other EPE, CTX-M-producing strains sometimes present low MICs for ceftazidime (Pitout et al. 2005, I), and successful ceftazidime treatment in patients with CTX-M-producing *E. coli* featuring in vitro susceptibility has been described (Pitout and Laupland 2008). There are also reports indicating that high doses of cefepime could be effective (Lee et al. 2007). Although cephalosporins have been associated with the selection of ESBL-producing strains, they remain well tolerated, non-toxic, bactericidal antimicrobials, well suited for empirical treatment of severe infections. Although ESBLs usually lead to cephalosporin resistance, the cephalosporins resist hydrolysis by β -lactamases better than penicillins. This make cephalosporins easier to protect with β -lactamase-inhibitors (Shlaes 2013). Therefore, ceftazidime and other cephalosporins combined with new β -lactamase-inhibitors with broader spectrum than existing inhibitors, have emerged as promising treatment alternatives for EPE. One example of such new inhibitor is avibactam (NXL-104) that can inactivate KPC carbapenemases. Novel cephalosporins are also being developed and combined with old β -lactamase-inhibitors.

3.2.3 Ceftolozane-tazobactam (CXA-201)

The novel cephalosporin ceftolozane (former CXA-101) has a broad gram-negative spectrum with activity against carbapenem-resistant and cephalosporin-resistant *Pseudomonas aeruginosa*. Human phase 1 studies of CXA-101 have shown a favourable safety and predictable pharmacokinetic profile with high target attainment for infections in the urinary tract (Ge et al. 2010). Also, human phase 2 studies have been completed for CXA-101 against complicated urinary tract infection, and the microbiological and clinical outcomes were comparable to ceftazidime (Cubist, data

not published). However, ceftolozane can easily be hydrolysed by ESBLs and has therefore been combined with tazobactam. Ceftolozane/tazobactam (CXA-201) is being developed as a first-line intravenous therapy for the treatment of serious Gram-negative infections caused by multi-resistant *Enterobacteriaceae* and *P. aeruginosa*, including EPE. In paper II, which was a collaboration with the pharmaceutical company Calixa Therapeutics (later acquired by Cubist Pharmaceuticals), we studied the in vitro activity of CXA-201 against our consecutive and largely polyclonal collection of 169 ESBL-producing strains. We found that the in vitro activity was very high, 88-98% depending on the concentration of tazobactam and the susceptibility breakpoint. The highest activity was seen with a high concentration of tazobactam (8mg/L) and the higher breakpoint (MICs were determined with broth microdilution) (Table 3). As previously described, the strain collection was relatively diverse according to PFGE (Fig. 7), and also comprised a similar fraction of CTX-M-15 and *E. coli* O25b-ST131 as reported from many other countries. Therefore we concluded that the results can probably be generalized to ESBL populations found in other regions of the world.

Table 3. Activity of ceftolozane + tazobactam and comparators against EPE (paper II).

Antimicrobial agent and concentration (breakpoint)	% Susceptible isolates
ceftolozane + tazobactam 4mg/L (1/4)	88
ceftolozane + tazobactam 8mg/L (1/4)	96
ceftolozane + tazobactam 4mg/L (2/4)	93
ceftolozane + tazobactam 8mg/L (2/4)	98
Ceftazidime + clavulanate	95
Ceftazidime + tazobactam	93
Piperacillin + tazobactam	58
Amoxicillin + clavulanate	24
Ampicillin + sulbactam	2

As shown in Table 3, ceftolozane/tazobactam performed much better than other β L/ β LI on the market (TZP, amoxicillin/clavulanate and ampicillin/sulbactam), and could therefore be an interesting option for isolates resistant to TZP. However, high susceptibility rates were also obtained with new combinations of old agents, ceftazidime/clavulanate and ceftazidime/tazobactam. To a clinician, these alternatives might seem more convenient to introduce compared to developing new cephalosporins. However, new combinations of old agents need to undergo the same clinical trials as novel agents, but are not associated with the same profit, and are therefore not attractive to evaluate by the pharmaceutical companies.

Although our study showed promising results for ceftolozane/tazobactam, other studies have now shown insufficient activity against ceftazidime-resistant *Enterobacteriaceae* and EPE populations in general with MIC₉₀ of 16 and >16 (Sader 2012). Ceftolozane/tazobactam is currently undergoing Phase III trials for complicated UTI and complicated intraabdominal infection. Whether ceftolozane/tazobactam is actually a future therapeutic option against EPE infections, or just a better pseudomonas-agent

similar to ceftazidime when it comes to treating other Gram-negatives, remains to be seen.

3.3 OTHER PARENTERAL AGENTS

Apart from the carbapenems and β L/ β LI, there are several parenteral agents that have displayed high activity in vitro, but for which there is very little clinical data to support their effectiveness as empirical as well as definite treatment against EPE.

Aminoglycosides (AGs) inhibit protein synthesis by binding to the 30S subunit of the ribosome, and have good effect against Enterobacteriaceae, although the use is being limited by their nephrotoxic side-effects (Mingeot-Leclercq and Tulkens 1999). As mentioned before, co-resistance to AGs is frequent among CTX-M-15 producing *Enterobacteriaceae* through the plasmid-mediated aminoglycoside-modifying enzyme *aac*(6')-Ib-cr (Oteo et al. 2010). Other *aac*(6')-I-subclasses inactivate amikacin but not other AGs, but *aac*(6')-Ib-cr causes partial cross-resistance between different AGs (Maurice et al. 2008, Hanberger et al. 2013). There are also several other mechanisms for AG-resistance, such as reduced transport through the cell, methylation of RNA and increased activity of efflux-pumps of which the last affects the various AGs differently. Among ESBL-producing isolates, resistance to amikacin is less common than to gentamicin and tobramycin, which was also true for our isolates (x% vs Y%) (I). In Stockholm, gentamicin is the most commonly used AG, followed by netilmicin, while amikacin is mostly used against tuberculosis. This might have contributed to the high resistance to gentamicin. Generally, and also in our setting, amikacin is preferred for the treatment of ESBL-infection (Hanberger, et al. 2013) which is currently being acknowledged and implemented in the hospitals of Stockholm.

In times of increasing antimicrobial resistance, old and "forgotten" agents are being re-evaluated. One example of an old antimicrobial that is being re-examined is **temocillin**. Temocillin is a ticarcillin derivative that (like other β -lactams) bind to penicillin-binding proteins and inhibit cell wall synthesis. It acts only against Enterobacteriaceae (van Duin et al. 2013). Temocillin is only available in Belgium and the UK, where it has the indications BSI, UTI and lower respiratory tract infections. Temocillin has a high stability against various β -lactamases, including extended-spectrum TEM, SHV and CTX-M enzymes (Livermore and Tulkens 2009). With the increasing β -lactamases among Gram-negatives, this is being appreciated, and temocillin has been reported to retain good in vitro activity against EPE (Livermore et al. 2006). We found fairly high susceptibility rates in our collection of EPE (I) although lower than previous reports (Livermore et al. 2006) and somewhat lower for the most common ESBL genotype CTX-M-15 (70%). Considering that the clinical effect against EPE is not well studied, the susceptibility rate against CTX-M-15-positive isolates may not be good enough to motivate an introduction of the agent on the Swedish market. However it might be an option to consider in the future, especially for pyelonephritis (Livermore and Tulkens 2009), where the effect is best shown for non-ESBL producing isolates.

Tigecycline is a new tetracycline derivative (glycylcycline). Tigecycline is bacteriostatic and acts by binding to the 30S ribosomal subunit and inhibiting protein synthesis. It is approved (in Sweden) for the treatment of complicated

intraabdominal, soft-tissue- and skin-infections. Tigecycline has shown excellent in vitro activity against *E. coli*, including CTX-M-15 isolates (Pitout 2010, I), whereas *Klebsiella spp.* can easily develop resistance due to efflux (Garau 2008). Despite its high in vitro activity against EPE, tigecycline has limited urinary excretion and is therefore not suited for the treatment of UTIs (Kanj and Kanafani 2011) which constitute the majority of ESBL-infections. In its favor is the minor impact of tigecyclin on the normal human microflora (Rashid et al. 2012). However, the use of tigecycline has been associated with increased mortality in clinical studies (van Duin et al. 2013). This has been explained by insufficient antibacterial effect probably related to high intracellular accumulation and low serum concentrations. Tigecycline should therefore be used only when no other options are available.

3.4 ORAL AGENTS

With the rapid increase in community-acquired UTIs caused by EPE, the lack of available oral antimicrobial agents with activity against ESBL is a matter of concern. The high level of co-resistance to fluoroquinolones and trimethoprim has compromised these alternatives, and data on the clinical outcomes for other oral agents against ESBL-producing bacteria are scarce. As shown in Table 2 the *E. coli*-isolates investigated in paper I retained high susceptibility to three oral agents: fosfomycin, nitrofurantoin and mecillinam. Nitrofurantoin and pivmecillinam constitute the two first-line therapies for uncomplicated lower UTI (cystitis) recommended by the Swedish National Health Institute (Infektionsläkarföreningen 2006). The majority of the Swedish EPE-isolates display low MICs to both of these agents, and although data on their clinical efficacy against EPE isolates are lacking, we are using them against lower UTI also when caused by EPE. However, fosfomycin is considered the safest choice against cystitis caused by ESBL-producing *E. coli* and *K. pneumoniae*, regardless of ESBL genotype (Garau 2008, Falagas et al. 2010).

3.4.1 Fosfomycin

Fosfomycin is a phosphonic acid derivative that is bactericidal against a broad spectrum of Gram-positive and Gram-negative organisms, by inhibition of bacterial cell wall synthesis. Fosfomycin is currently available in many countries both in oral and intravenous forms. Oral fosfomycin should be restricted to treatment of lower UTI and has become first-line therapy for cystitis in several European countries with high prevalence of EPE (Falagas, Kastoris et al. 2010, Falagas, Vouloumanou et al. 2010). Intravenous fosfomycin is used against more severe infections of various types (van Duin et al. 2013). Resistance to fosfomycin has previously been very low in *E. coli*, and related to chromosomal mutations. However, acquired resistance among CTX-M-15-producing ST131-isolates has been described and seems to be emerging. A Spanish study recently reported an increase in fosfomycin resistance in urine cultures with EPE from 0 to 14.4% between 2005 and 2011 (Rodriguez-Avil et al. 2013). Fosfomycin was taken off the Swedish market in 2009 because of the low use. Ironically this happened just when we were entering the ESBL-era and the agent suddenly had a new important indication. A re-introduction would be welcome, since there are very few oral alternatives against ESBL-UTI.

3.4.2 Nitrofurantoin

Nitrofurantoin is an antibiotic with low tissue distribution and high renal excretion, suitable for the treatment of lower UTI caused by *E. coli*. The effect is bacteriostatic at low and bactericidal at high concentrations. Although clinical data on nitrofurantoin against EPE are far from extensive, the agent has been reported effective in the treatment of lower UTI (Tasbakan et al. 2012). In Sweden we still have very low resistance rates despite a tradition of relatively high use. However, with increasing resistance to trimethoprim, trimethoprim has lost its place in the first-line therapy against lower UTI in favor of nitrofurantoin, and it remains to be seen how this will affect the resistance. In the mean time we are using it against lower UTI regardless of ESBL-production, because of high in vitro activity (I). In cases of treatment failure against EPE, pivmecillinam treatment may be an alternative.

3.4.3 Pivmecillinam

Pivmecillinam is an amidinopenicillin with selective and high activity against Gram-negative organisms, especially *E. coli*. It is well documented that pivmecillinam is effective and well tolerated for the treatment of acute uncomplicated cystitis in women (Nicolle 2000) and long-term clinical experience from the Nordic countries supports its efficiency. As it is eliminated through the kidneys, very low concentrations are attained in feces and the impact on the fecal flora is therefore minor (Sullivan et al. 2001). However, pivmecillinam is not available in many countries outside Scandinavia. The in vitro activity of mecillinam is high against ESBL-producing isolates (I) and pivmecillinam could therefore represent a treatment alternative for lower UTI caused by EPE also in countries that traditionally have not had access to it. Apart from one case report suggesting that pivmecillinam could be effective against pyelonephritis caused by ESBL-producing *E. coli* (Nicolle and Mulvey 2007), there was no data on the clinical outcome for pivmecillinam against EPE before paper III. This paper was an attempt to evaluate the clinical and bacteriological activity of pivmecillinam against lower UTI caused by EPE further. The patients were selected from the cohort of patients with first time EPE-infection in paper IV (n=61). Patients with lower UTI that had received pivmecillinam as single-therapy and whose urine isolates were retrievable (n=8) were included. The patients who had received other agents (n=9) were included as a control group. The study outcome was antimicrobial efficacy based on bacteriological and clinical cure. Bacteriologic cure was defined as $<10^3$ CFU/ml at the 30 days follow-up. Patients were considered clinically cured if the urinary symptoms had resolved after completed treatment according to the medical record. Relapse of UTI was defined as symptoms of acute cystitis, and/or signs of upper UTI, presenting within 6 months after clinical cure. We found that all patients receiving pivmecillinam had good clinical response; however, the bacteriological cure rate was low (2/8). Interestingly, none of the patients with persisting bacteriuria after 30 days had a relapse of UTI within 6 months, whereas one of the patients with bacteriological cure did. The results are displayed in Table 4 (controls not shown). There was no indication that the received dose of pivmecillinam, in the range of 200 mg twice daily (b.i.d, i.e., bis in die, latin) to 200 mg three times daily (t.i.d, i.e., ter in die, latin), affected the clinical or the bacteriological outcome. However, none of the patients received 400 mg t.i.d..

The use of pivmecillinam is recommended as treatment for uncomplicated cystitis (Gupta et al. 2011), but not against complicated UTI. Half of the patients in our study could not be classified as uncomplicated as they had functional or structural abnormalities of the genitourinary tract (9/17 in total; 4/8 who received pivmecillinam). It is well known that patients with structural or anatomical problems within the urinary tract are less likely to experience bacterial eradication after infection. It is possible that the high rate of persisting bacteriuria in our collection to some extent reflects the underlying complicating factors of the patients, which could explain the low bacteriological cure. In these cases the selection of an antimicrobial for clinical therapy should be individualized considering patient tolerance, clinical presentation, recent prior antimicrobial treatment, prior urine culture results, and known or suspected susceptibilities, and we do not know if this was done. However, the importance of persisting bacteriuria can be questioned when clinical cure is achieved and relapse does not occur. Another fact to take into account in this small study is spontaneous cure of uncomplicated cystitis, which according to a Swedish clinical trial comparing pivmecillinam with placebo, may occur in about 20% of all patients (Ferry et al. 2007). However, this may only apply to the patients without underlying complicating factors and is therefore of less relevance in our study. Further, in the same study all pivmecillinam dosing regimens were superior to placebo.

Although the number of patients in this series of case-reports is limited, our results give a clear indication that pivmecillinam has good clinical activity against lower UTI with EPE, but that the bacteriological activity is questionable, at least with the current dosage regimens in patients with underlying complicating factors. Potentially, this agent could also have a place in the antimicrobial armamentarium in countries where it has not previously been licensed. However, our data also suggest that persisting bacteriuria is of little clinical importance, although larger clinical studies are needed to confirm this.

Large prospective comparative trials to evaluate the clinical activity of nitrofurantoin and pivmecillinam against EPE UTI are needed. These studies are difficult to perform in low-endemic countries like Sweden because of the very high number of patients that would have to be included in order to find enough patients with EPE.

Table 4. Women with cystitis: susceptibility and response to pivmecillinam treatment, and risk factors for ESBL (modified from paper III).

Species (CTX-M- group)	Pivmecillinam dosage regimen ¹	Susceptibility (MIC mg/L/zone dia mm) ²	Bacteriological cure ⁴	Clinical cure	Relapse of UTI within 6 months	Risk factors for ESBL infection					
						Additional antimicrobial treatment ³	Hospitalization ⁶	Previous antimicrobial treatment ^{4,5}	Urinary catheter ⁵	Abnormality in urinary tract	Travel abroad ⁵ (country)
<i>E. coli</i> (CTX-M-1)	200mg t.i.d. 5d	S (0.25 / 25)	YES	YES	NO	-	-	-	-	-	Cambodia
<i>E. coli</i> (CTX-M-1)	Data missing	S (1 /18)	NO	YES	NO	AMX	-	-	-	-	Italy
<i>E. coli</i> (CTX-M-9)	200mg b.i.d. 7d, 400mg bid 7d	S (0.25 /28)	NO	YES	NO	-	-	Unknown agent (cystitis)	YES	Recurrent cystitis	-
<i>E. coli</i> (CTX-M-9)	200mg b.i.d. 7d	S (0.5 /22)	NO	YES	NO	AMX	-	AMX, DOX	-	Inconti- nence	-
<i>E. coli</i> (CTX-M-1)	200mg t.i.d. 7d	S (2 / 17)	NO	YES	NO	-	YES	FLU, NIT	-	-	-
<i>E. coli</i> (CTX-M-1)	200mg t.i.d 7d	S (0.25 /25)	NO	YES	NO	-	YES	Unknown agent	YES	MS, chronic catheter	-
<i>E. coli</i> (CTX-M-8/25)	200mg b.i.d. 7d	S (0.25 / 26)	NO	YES	NO	-	-	-	-	-	Spain
<i>K. pneumoniae</i> (CTX-M-9)	200mg b.i.d. 7d	S (0.5 /23)	YES	YES	YES (After 5 weeks)	-	YES	PcV	-	ESWL -08	-

¹B.i.d (bis in die, latin) = twice daily; t.i.d (ter in die, latin) = three times daily²MIC-breakpoints according to EUCAST (zone diameter breakpoint) (S≤/R>): MEC 8/8 (15/15)³<1,000 CFU/mL, urine sample 1 month after initial culture⁴AMX=amoxicillin; DOX=doxycycline; FLU=flucloxacillin, PcV= fenoxymetyl penicillin, MZL=metronidazole⁵ within 6 months before infecti

4 FECAL CARRIAGE OF ESBL-PRODUCING ENTEROBACTERIACEAE

Fecal carriage of EPE among asymptomatic individuals is increasing. Colonizing bacteria may serve as a source for later infection, and therefore will affect the choices of empirical antimicrobial treatment. In order to limit the use of inappropriate empiric antimicrobial drug therapy, we need to define patients at risk for fecal carriage of EPE. Thereby we can limit the mortality in patients with severe infections caused by EPE. Also in non-severe infections it is critical to identify those with EPE-carriage in order to prevent and/or limit outbreaks. To address this need, prediction tools based on risk factors for EPE-infection are being created to identify patients harboring EPE organisms (Johnson et al. 2013). In this context it is not only important to define patients at risk for carriage of EPE but also to predict how long the ESBL-carriage will persist and identify patients at risk for prolonged carriage. Very little is known about the duration of ESBL-carriage as well as host and pathogen factors associated with prolonged carriage. These aspects of EPE-carriage were studied in paper IV.

4.1 THE NORMAL FECAL FLORA

The human colon contains 10^{12} bacteria per gram of contents and >100 bacterial species. Major functions of these bacteria are metabolic activities that result in salvage of energy and absorbable nutrients, trophic effects on the intestinal epithelia and protection against potentially pathogenic microorganisms (Guarner and Malagelada 2003). The gut flora prevents the colonization of exogenously introduced organisms but also avoid the overgrowth of already present pathogens such as certain strains of *E. coli*. There are several mechanisms that may contribute to inhibition of pathogens, e.g. prevention of access to adherence sites in the mucosa, production of inhibitory substances or conditions and reduction of nutrients. The specific bacteria that are the most important in this defense are not known, although studies have suggested that obligate anaerobes are crucial (Donskey 2004).

4.1.1 *Escherichia coli*

E. coli is the most prevalent facultative aerobe in the feces of humans (Donskey 2004). Most strains do not cause disease. However, *E. coli* is also an important pathogen, being the most common cause of UTI. *E. coli* may also cause abdominal, airway, wound and blood stream infection as well as diarrhoea and meningitis. Uropathogenic strains of *E. coli* express certain properties, products or structures referred to as virulence factors (VFs). VFs help the organism to overcome host defences and colonize or invade the urinary tract. VFs associated with UTI include adhesins (P fimbriae and type 1 fimbriae), the aerobactin system, hemolysin, K capsule and resistance to serum killing (O-antigen) (Johnson 1991). Fig. 11 shows how these VFs can interact with a host cell to cause infection and Table 5 lists their main functions.

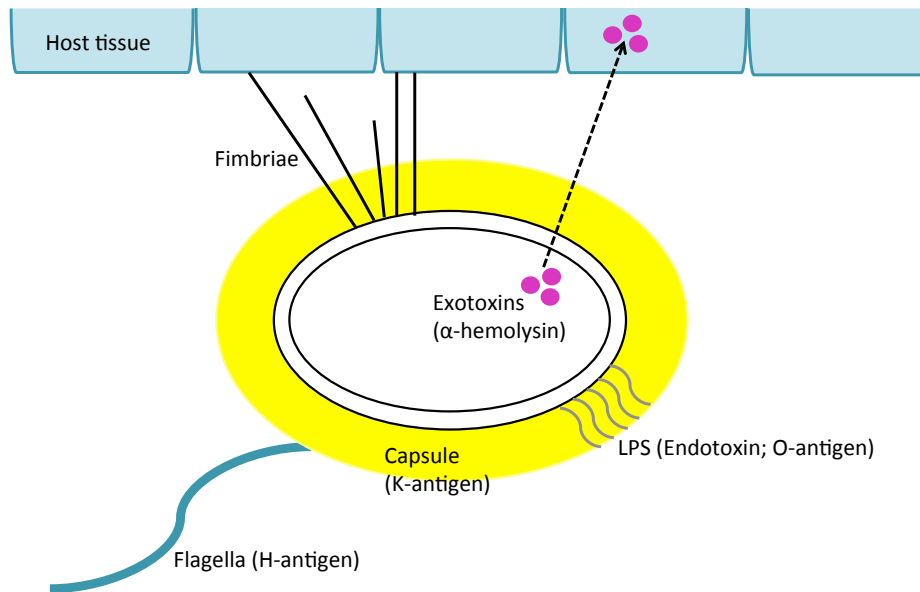


Figure 11. Schematic overview of an *E. coli* interacting with a host cell. Attachment is the first necessary step in the colonization of a host's mucosal surfaces and is facilitated by fimbriae that also stimulate inflammation. The exotoxins secreted by the bacterium facilitate invasion. The K-antigen helps the bacteria to avoid phagocytosis by the immune-system and helps the O-antigen promote serum resistance and avoid lysis. (Most of the picture adapted from Johnson 1991).

Table 5. Major VFs of uropathogenic strains of *E. coli* and their functions

Virulence factor	Effect*
P-fimbriae	Adhesion to host tissue Stimulates inflammation
Type-1-fimbriae	Adhesion to the mucosa Renal scarring
Aerobactin	Promotes bacterial growth
Hemolysin	Toxicity to host tissue Disruption of phagocytosis Release of iron from erythrocytes
K-antigen	Avoidance of phagocytosis Promotes serum resistance
O-antigen	Promotes serum resistance

* (Johnson 1991)

In the clinical microbiological laboratory, all *E. coli* share the same features of the species, although they may express different virulence factors. Unless these factors are looked for by molecular methods, one will not be able to distinguish between pathogenic and non-pathogenic *E. coli*.

E. coli can be divided into six phylogenetic groups (A-E) and/or be classified by serotype, based on differences in their O (lipopolysaccharide), H (flagellar protein), and

K (capsula) antigens. Although these classification systems cannot predict virulence, phylogroups B2 and D have been associated with high virulence (Wold, Caugant et al. 1992). Strains within the same serotypes and/or phylogroups can be distinguished by genomics.

4.1.2 *Klebsiella pneumoniae*

K. pneumoniae is an encapsulated and non-motile bacteria. It is the clinically most significant of the *Klebsiella* spp. and infections occur in humans of all ages although the highest risk groups appear to be infants, the elderly and the immunocompromised. *K. pneumoniae* is associated with hospital acquired infections such as catheter related UTI and ventilator associated pneumonia (VAP) but is also the second causative agent of UTI in the elderly. *K. pneumoniae* express O-antigens and K-antigens on their surface which, as for *E. coli*, contribute to pathogenicity and form the base for serogrouping. Serogrouping or screening for virulence genes was not performed within this thesis (Schaechter 1993, Mandell 2000).

4.2 PREVALENCE AND RISK FACTORS FOR EPE-CARRIAGE

We know that asymptomatic fecal carriage of EPE is increasing rapidly but it is very difficult to estimate how wide-spread carriage of EPE is in entire populations. However, there is a number of reports on the prevalence rates of fecal ESBL-carriage from different parts of the world. Asymptomatic carriage among healthy individuals has been reported as 65.7% from Thailand (Luvsansharav et al. 2012) and 6.7% from Spain (Oteo 2010). In a study from the Netherlands of community patients with gastrointestinal complaints 10.1% were ESBL-positive (Reuland et al. 2013). A British study by Wickramasinghe et al showed a point prevalence rate of 11.3% in fecal samples from community patients, and that the prevalence was significantly higher (22.8%) in patients with an origin in South Asia or the Middle-East (Wickramasinghe et al. 2012). In children attending a pediatric ward in Guinea Bissau the prevalence was 32.6% (Isendahl et al. 2012). There are also a few Swedish studies reporting an EPE-prevalence in feces of 3% of primary care patients and 7% among hospitalized patients in Skåne (Stromdahl et al. 2011), and 5% in patients admitted for abdominal surgery in Linköping 2006-2007 (Chabok et al. 2010). In healthy pre-school children in Uppsala 2.9% were ESBL-positive (Kaarme et al. 2013) and so were 3% of residents of nursing homes in Stockholm (Andersson et al. 2012).

Some parts of the world are considered high-risk areas, and travel to these areas such as the Indian subcontinent and the Middle East, is a major risk factor for acquisition of asymptomatic fecal carriage of EPE (Tangden et al. 2010, van der Bij and Pitout 2012). As discussed previously, the high acquisition among travellers is largely linked to the internationally spread *E. coli* clone ST131, carriage which may occur through consumption of contaminated food and water (Peirano and Pitout 2010). In endemic regions previous healthcare contact and antimicrobial exposure have been identified as risk factors for asymptomatic carriage (Luvsansharav et al. 2012). Contact with healthcare centres and previous use of antimicrobial agents including cephalosporins and fluoroquinolones as well as comorbidities (renal and liver pathology, diabetes mellitus) are also well known risk factors for community-onset infections with EPE (Ben-Ami et al. 2009, Oteo et al. 2010). The spread within hospitals is facilitated if the

EPE-carrying patient has diarrhoea, urinary catheter or other type of catheters and drains and of course low infection control standards. However, with the increasing number of EPE-carriers, transmission of EPE within households has been found to outweigh nosocomial dissemination in the non-outbreak setting (Hilty et al. 2012).

4.3 DURATION OF CARRIAGE

Although the prevalence and risk factors for fecal carriage with EPE have been studied, little is known about the time course of stool colonization of EPE. One study from Thailand shows that the fecal carriage often persists for at least three months and that antibiotic treatment may prolong the carriage (Apisarnthanarak et al. 2008) and results from a Slovenian study indicates that 51.5% were carriers after six months (Papst L. 2012). Warren et al. found 20% of the patients to be carriers one year after infection, but less than 5% after two years (Warren et al. 2008). In a smaller Swedish study 10/41(24%) patients (24%) carried EPE after 3-8 months (Tham et al. 2012). A recent Israeli study of the duration of carbapenem resistant Enterobacteriaceae (CRE) showed that mean time to CRE negativity was 387 days (95% confidence interval: 312-463). Seventy-eight percent of patients (64/82) had positive culture at 3 months, 65% (38/58) at 6 months, and 39% (12/30) at 12 months (Zimmerman et al. 2013). In a Norwegian study of infants colonized during a nosocomial neonatal intensive care unit outbreak and their household contacts, the median carriage time among the infants was 12.5 months, although only 2.5 months among their parents (Lohr et al. 2013). A possible explanation for this is that the fetal intestine is sterile and that colonizing bacteria during the first days of life influences the composition of the intestinal flora (Westerbeek, van den Berg et al. 2006) and leads to persistence of the colonizing bacteria. Paper **IV**, which presents prospective data on the duration of fecal carriage of ESBL after first time infection caused by an EPE (n=61), showed that 66% were carriers after three, 55% after six and 44% after twelve months. The main limitation of this study is the fairly small number of patients. However, the mean age, sex and culture material did not differ from the total group of patients diagnosed with clinical ESBL-infection during recruitment (n=522) as shown in Table 6, indicating that the results should be generalizable and relevant for most patients with ESBL-infection.

In accordance with previous studies (Alsterlund et al. 2012, Lohr et al. 2013) we found that among EPE-carriers twelve months after EPE-infection it is not uncommon with one or several negative fecal samples within the first year of carriage (**IV**, ref). It is possible that EPE persists in the gut but is suppressed by the normal intestinal flora enough not to be detected by routine methods, and that ecological disturbances caused by e.g. antimicrobial treatment on a later occasion promotes overgrowth of the resistant bacteria. However, in our material (**IV**) there was no correlation between antibiotic intake at any stage of the follow-up and a shift from negative to positive faecal samples. Three consecutive negative samples have previously been suggested to define elimination of carriage (Alsterlund et al. 2012), although our data indicates that three samples may be too few.

Table 6. Characteristics of 508 patients with EPE detected at the clinical microbiology laboratory at Karolinska University Hospital between February and December 2009¹ (paper IV).

	All patients (n=508)	Patients included in analyses (n=61)	Patients not included in analyses (n=447)	P-value
Female	337 (66)	38 (61)	299 (67)	0.48
Age (mean)	57.8	58.3	57.7	0.87
<i>Level of care</i>				0.002
Hospital inpatients	308 (60)	35 (57)	273 (61)	
Hospital outpatients	50 (10)	14 (23)	36 (8)	
Long term care facilities	25 (5)	0	25 (6)	
Primary care	125 (25)	12 (20)	113 (25)	
<i>Culture material</i>				0.39
Urine	437 (86)	51 (84)	386 (86)	
Blood	24 (5)	5 (8)	19 (4)	
Other	47 (9)	5 (8)	42 (9)	

¹Data presented as number and column percentage in parenthesis if not otherwise stated.

4.3.1 Factors affecting the duration of carriage

Very little is known about how host and pathogen factors affect the duration of carriage of EPE. Previous studies have suggested that antimicrobial treatment will prolong carriage (Apisarnthanarak et al. 2008, Lohr et al. 2013), and studies of CRE has shown that multiple hospitalizations and CRE disease (as opposed to screening samples) extend the duration of carriage (Schechner et al. 2011, Zimmerman et al. 2013). Abnormalities within the urinary tract have also been associated with persisting carriage of KPC *K. pneumoniae* (Feldman et al. 2013). In infants, apart from antimicrobial treatment, caesarean section has been found to prolong carriage of EPE (Lohr et al. 2013). Caesarean section is known to influence the development of the intestinal microbiota (Westerbeek et al. 2006), which could explain the association. We found strain factors related to high virulence to be associated with persisting carriage of EPE, whereas antimicrobial treatment and other patient-related factors were not (Table 7) (IV). These findings are being discussed further below.

Table 7. Comparison of patient and strain factors between ESBL-carriers and non-carriers twelve months after EPE-infection (modified from paper IV).

	Variable	Carriers at 12 months n(%)	Non-carriers at 12 months n(%)	P-value
Sex and age	Women	14 (54)	21 (60)	0.8
	Median age (years)	63	60	0.9
Type of infection	Urinary tract infection	24 (92)	30 (86)	0.7
	Blood stream infection¹	6 (23)	1 (3)	0.04
Risk factors for acquisition of EPE	Previous antimicrobial ²	10 (42)	16 (47)	0.8
	Hospital stay ²	13 (52)	14 (41)	0.4
	Travel abroad ²	11 (31)	20 (60)	0.3
	Abnormalities urinary tract	5 (20)	8 (24)	0.76
Antimicrobial treatment ³	Carbapenem treatment	5 (19)	2 (3)	0.15
	CIP + TMP +TSU ⁴	9 (39)	11 (52)	0.8
	NIT + MEC ⁴	7 (30)	11 (52)	1.0
	>2 antimicrobial agents	3 (13)	5 (16)	1.0
	>3 antimicrobial agents	2 (9)	2 (6)	1.0
Strain factors	Phylogroup B2	12 (52)	6 (23)	0.04
	ST131	9 (39)	5 (19)	0.2
	CTX-M-1-group	15 (60)	23 (74)	0.4
	CTX-M-9-group	10 (40)	3 (10)	0.01
	<i>fim</i>	23 (100)	25 (96)	1
	<i>pap</i>	14 (7)	3 (12)	0.7

¹ Bold face=P<0.05.

² Six months before ESBL-infection

³ For the original infection with ESBL-producing bacteria (not additional treatment during follow-up)

⁴ CIP=ciprofloxacin, TMP=trimethoprim, TSU=trimethoprim-sulfamethoxazole, NIT=nitrofurantoin, MEC=mecillinam

4.3.1.1 *The virulence of the bacteria*

E. coli strains differ widely in colonization capacity in the colon, and this capacity is linked to virulence factors in extraintestinal infection (Wold et al. 1992). Strains belonging to the phylogenetic group B2 and D have an enhanced ability to persist in the intestinal flora (Wold et al. 1992). Genes encoding P fimbriae and other virulence factors associated with uropathogenicity such as type 1 fimbriae, hemolysin, capsular polysaccharides and aerobactin are enriched in resident strains, and there seems to be an additive effect of these factors promoting persistence (Wold et al. 1992, Melican et al. 2011, Ostblom et al. 2011). In accordance with previous studies on non-ESBL isolates, we found that the *E. coli* phylogenetic group B2 was associated with prolonged carriage of EPE, which was also the case for CTX-M-gr.-9 (IV). Of the eleven CTX-M-gr.-9-positive *E. coli* isolates, nine belonged to phylogroup B2 or D (4 and 5 isolates respectively) indicating that these strains were highly virulent. Although B2 is strongly associated with high virulence, we did not find any differences in the presence of the *pap*- and *fim*-genes encoding the uropathogenic virulence factors p-fimbriae (*pap*, pili associated with pyelonephritis) and type 1-fimbriae (*fim*) between carriers and non-carriers. On the contrary, almost all infectious isolates were positive for *fimH* and *fimAMT78*-genes, probably reflecting the fact that infectious isolates of *E. coli* are virulent and mostly uropathogenic by definition. Our findings still indicate that strain virulence could be an important factor also for persistence of ESBL-producing *E. coli* in the intestinal microbiota.

We also found that CTX-M-gr.-9 was correlated to prolonged carriage. This ESBL genotype is perceived to be associated with less virulent strains than CTX-M-gr.-1. CTX-M-gr.-1 includes the CTX-M-15 enzyme associated with the highly virulent international clone ST131 and indirectly to phylogroup B2. Previous studies demonstrate that virulence of *E. coli* ST131 is related with the strain and not with the presence of *bla*_{CTX-M-15} (Rogers et al. 2011). Of the 11 CTX-M-gr.-9 *E. coli* isolates 9 belonged to phylogroup B2 or D (4 vs. 5 isolates), indicating that these strains were highly virulent which might explain the association with prolonged carriage.

4.3.1.2 *Antimicrobial treatment*

Antimicrobial treatment may suppress the commensal bacteria and facilitate for colonizers and pathogens to find an ecological niche. There is a wide variability in the ecological effect of antimicrobial agents. The factors of most importance for ecological disturbances are the antibacterial spectrum, degree of absorption, route of elimination, enzymatic inactivation and/or binding to human fluids and intestinal material (Sullivan et al. 2001). For example, trimethoprim-sulfamethoxazole and ciprofloxacin, show high concentrations attained in faeces and an important effect on the intestinal flora, while pivmecillinam and nitrofurantoin, agents that are mainly eliminated through the kidneys, are considered to account for minor impact (Sullivan et al. 2001). The carbapenems have the broadest spectrum of the antibiotics discussed in this thesis, and are therefore associated with considerable ecological disturbances.

It is possible that the use of antimicrobial agent(s) did affect the duration of carriage noted in paper IV. However, we did not find that the number of received antimicrobial agents was correlated to the duration of carriage and neither were there any differences between carriers and non-carriers at 12 months for specific agents. There was a trend that treatment with carbapenems was more frequent in patients with prolonged carriage (19% vs. 3%), although the difference was not statistically significant (p-value: 0.15). The use of carbapenems is associated with BSI, which was associated with prolonged

carriage in univariate analysis although not in multivariate analysis. However, *E. coli* strains belonging to the virulent phylogroup B2, were more common among patients with BSI. Thus, an association between BSI and/or carbapenem use and persisting carriage (although not shown in our study) could actually be an effect of strain virulence. Larger studies are needed that assess the effects of usage of different antibiotic classes on carriage duration. These should also take into account possible confounding factors since it is likely that antibiotic usage is associated with host factors such as co-morbidities.

4.3.1.3 Patient factors

Underlying abnormalities within the urinary tract are well known risk factors for acquisition of ESBL (Oteo et al. 2010), and have also been associated with persisting carriage of KPC *K. pneumoniae* (Feldman et al. 2013), but were in our study not associated with persisting carriage (IV). However, 6/13 (46%) patients with underlying conditions of the urinary tract did get a new ESBL-infection during follow-up compared to the overall rate 19/61 (31%). This could possibly be an effect of more complicated, persisting and/or frequent urinary tract infections, more frequent hospital stays and exposure to antimicrobials. Carbapenem treatment was also overrepresented among these patients (3/13 (23%) vs 6/61 (10%)), which might be an effect of more severe infection or more resistant strains due to previous antibiotics.

4.4 THE HOST STRAIN DURING EPE-CARRIAGE

4.4.1 Clonality and strain diversity

Subsequent generations of bacteria retain the parental DNA pattern until the DNA changes, which is known as clonality. Changes in the DNA through insertions, deletions and rearrangements lead to strain diversity. Within a clone, minor changes in the DNA (genetic events) over time are expected. There are various methods to determine whether different bacterial isolates are clonally related or diverse (epidemiological typing). In our studies epidemiological typing was performed with PFGE. PFGE is a high resolution method based on restriction enzymes cutting the entire bacterial DNA into various sized fragments. Changes in the DNA change the relative position of the restriction sites and thus change the length of the DNA fragments, which will be interpreted as strain diversity. However, when comparing isolates over time, as in paper IV, we cannot expect the PFGE-patterns of isolates belonging to the same strain to be indistinguishable when several or many months have passed. Some differences must be allowed.

Because ESBL-genes are transferable between bacteria it is likely that ESBL-enzymes, during fecal carriage, are transferred from colonizing strains to strains belonging to the normal intestinal microbiota. Therefore, in paper IV, PFGE was performed to determine whether the ESBL-production was found in the same or new strains for each patient during follow-up. We found the ESBL-production in another species and/or in a different or additional strain of *E. coli* or *K. pneumoniae* than in the beginning of the study in 28% of the patients (Fig. 12).

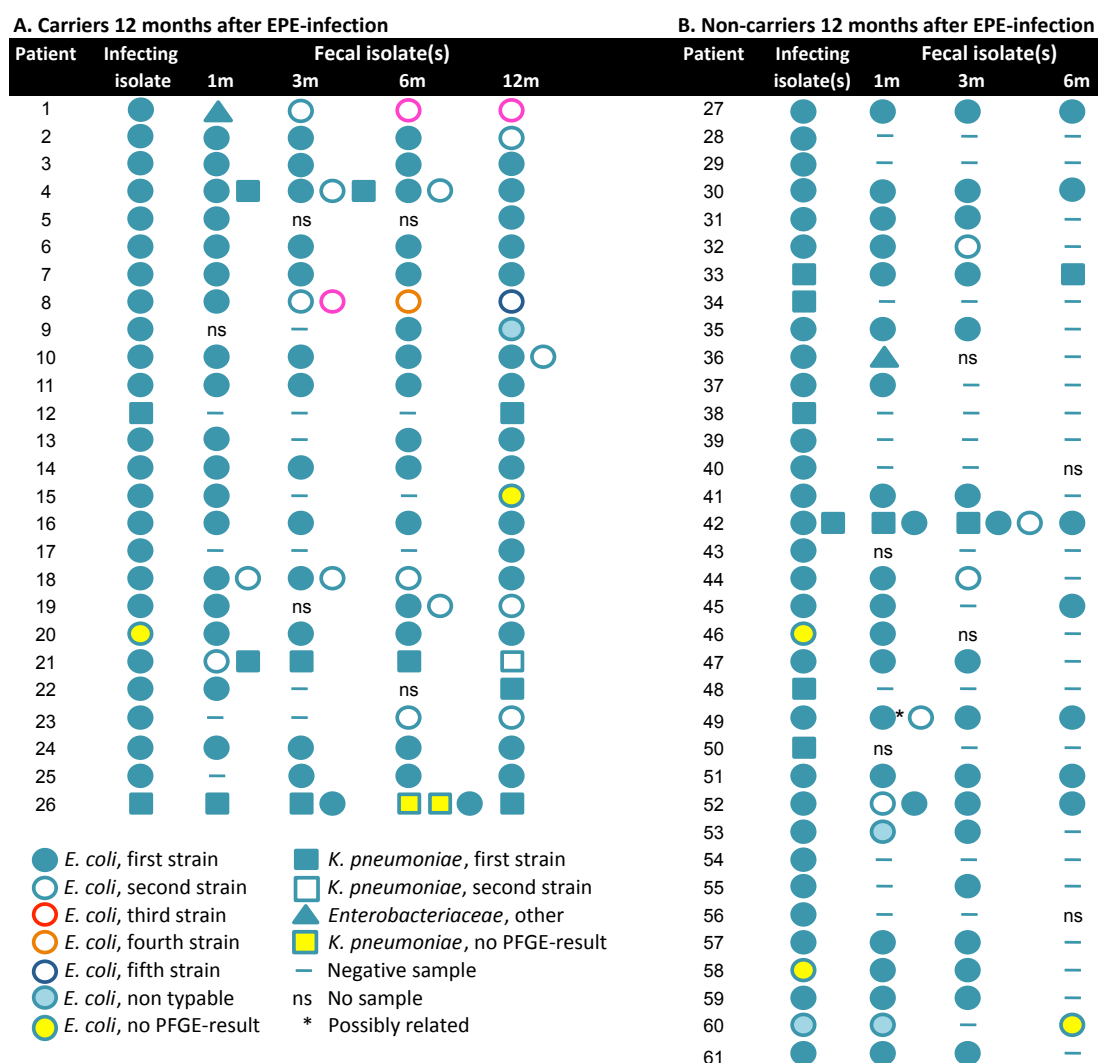


Figure 12. ESBL-producing strains during carriage. Overview of the duration and dynamics of fecal carriage. Fig. 12A displays results of cultures and PFGE for those who were carriers at 12 months, Fig. 12B for non-carriers. Each row shows the results for one patient. The symbols represent the species and strains where ESBL was found in the clinical sample and in feces at 1, 3, 6 and 12 months (paper IV).

This finding was more frequent among carriers at twelve months than non-carriers, supporting the hypothesis that persisting carriage is linked to transfer of ESBL-genes from infecting or colonizing strains to the normal fecal flora. However, nine of these patients had a new CTX-M-group in one or several of the follow-up samples, showing that the ESBL-production in these strains was not caused by the same ESBL-gene as in previous samples. The most probable explanation is that some of these patients were colonized with several strains in the beginning of the study, but that all the strains were either not present in all fecal samples or that we were unable to distinguish these as different strains when selecting colonies from the screening plates. Further, it cannot be excluded that some of these patients had acquired a new EPE isolate during the follow-up. Since there is little Swedish data on the prevalence of fecal carriage of ESBL in the community, it is very difficult to calculate the risk of being re-colonized. However, with the low prevalence of ESBL among clinical isolates of *E. coli* and *K. pneumoniae*

(<3%) (Struwe and Olsson-Liljequist 2009, Chabok et al. 2010, Stromdahl et al. 2011, Andersson et al. 2012) we find this less likely, provided that there had been no new exposure to risk factors such as hospital stay or travel to a high-risk country.

4.4.2 Plasmid transfer of ESBL-genes between different strains

ESBL-genes are often located on conjugative plasmids belonging to the IncF group, especially IncFII, as well as I1, N, and K, with high potential of recombination. A/C, H, L/M and P have also been found (Novais et al. 2007, Coque et al. 2008, Chouchani et al. 2012, Carattoli 2013). Our hypothesis was that during carriage of EPE, the plasmid harbouring the ESBL-gene is transferred from the colonizing strain to strains in the normal fecal flora. To study whether an ESBL-gene has been transferred through plasmid conjugation one needs to characterize the plasmids in each strain. One way to characterize plasmids is by grouping them into incompatibility (Inc) groups (Datta and Hedges 1971). To determine the Inc groups of the plasmids in the samples we used PCR-based plasmid replicon typing described by Carratoli et al, detecting the 18 major plasmid families for *Enterobacteriaceae* (Carattoli et al. 2005). In accordance with previous reports on EPE (Coque et al. 2008) we found that plasmids belonging to the IncF group were commonly detected in our isolates (FrepB (n=46), FIA (n= 29) and FIB (n=27)). These plasmids are frequently present in both ESBL- and non-ESBL-producing *E. coli* and *K. pneumoniae*, so their presence in two different ESBL-producing strains from the same patient does not confirm that an IncF plasmid transferred the ESBL gene. Further, it is quite common for isolates to have several plasmids, and one plasmid may contain multiple replicons. In this material FrepB, FIA and FIB were the most common replicon contents, and for the patients with ESBL-production in a different strain and/or species we found that in 4/17 cases the replicon content of the first and at least one of the new strains were identical. The results of the plasmid characterization are shown in Fig. 13.

We have not conducted experiments to confirm that ESBL was linked to the specific plasmids, and therefore have not specifically studied the replicon type of the plasmid carrying ESBL. The ESBL-containing plasmid could be of another replicon type than the one detected, although it is probable that they were associated. However, if two strains from the same patient carry plasmids with completely different Inc groups we can conclude that there has not been a plasmid transfer within the fecal flora of the patient, thus the new strain must have come from a different source. To confirm that the plasmids actually carry the ESBL-genes plasmid conjugation experiments would have to be performed. To confirm that two different plasmids carry the same DNA sequence that encodes the ESBL-production, restriction fragment length polymorphism (RFLP) could be used. To further characterize the plasmids carrying the ESBL-genes in our collection of strains, a collaboration with researchers at the Swedish Institute for Communicable Disease Control has been initiated.

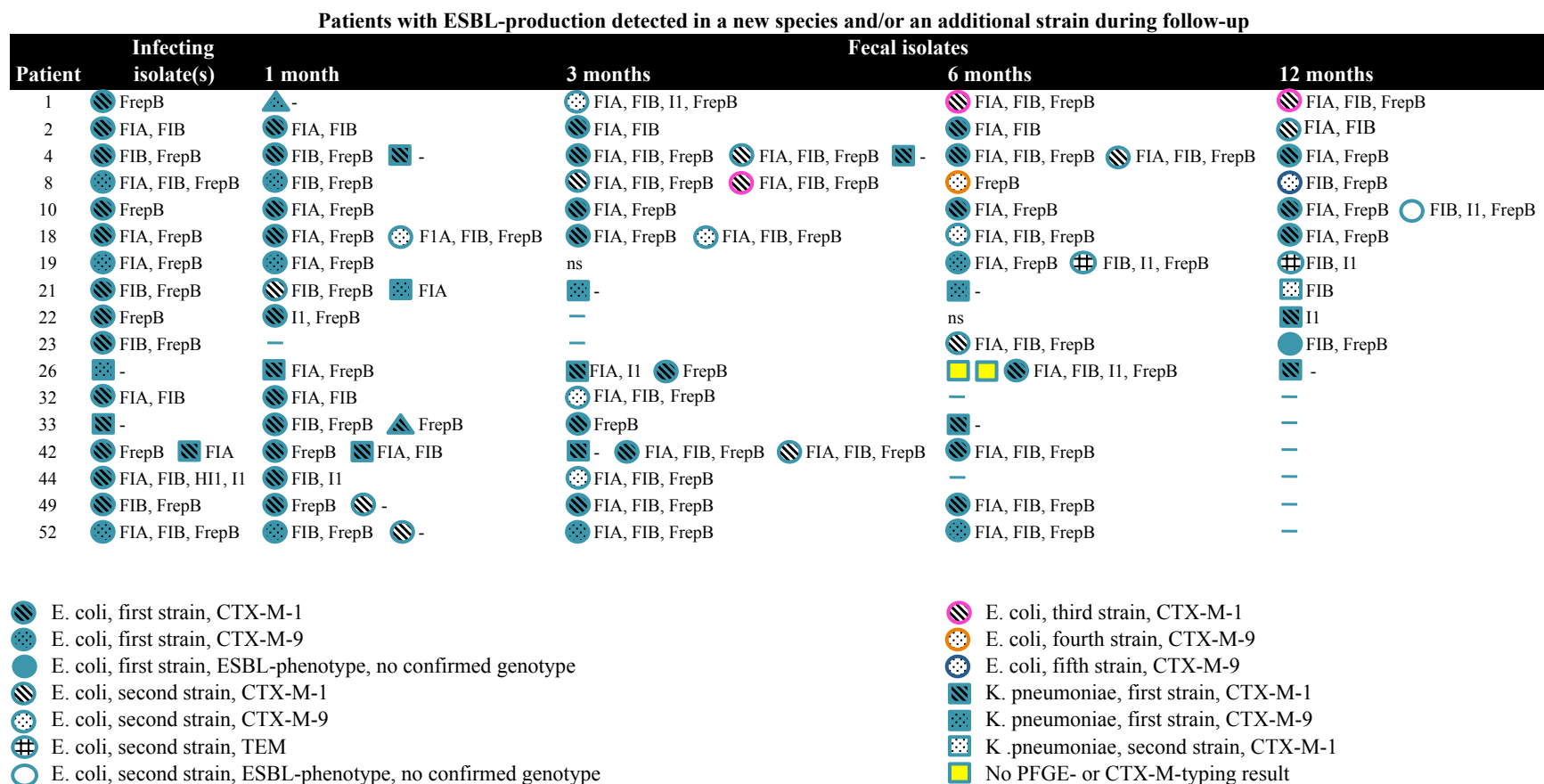


Figure 13. CTX-M-groups and replicon contents of ESBL-producing isolates from patients with ESBL-production detected in a new species or strain during carriage. Each row shows the results of cultures, PFGE and PBRT for one patient. The symbols represent the species and strains where ESBL was found in the clinical sample and in feces at 1, 3, 6 and 12 months (paper IV).

5 CONCLUSIONS

The papers included in this thesis provide insights on the epidemiology of ESBLs in Stockholm, knowledge on treatment alternatives to the carbapenems as well as on the duration of carriage of EPE. More specifically we show that:

- *bla*_{CTX-M-15} is the most common ESBL genotype in Stockholm.
- There are several therapeutic parenteral and oral alternatives to the carbapenems available for *E. coli* but few for *K. pneumoniae*.
- Ceftolozane-tazobactam has good in vitro activity against ESBL-producing Enterobacteriaceae and may be a future therapeutic option for infections caused by TZP- and ACL-resistant isolates.
- Pivmecillinam seems to have good clinical activity against lower UTI caused by ESBL-producing Enterobacteriaceae although bacteriological cure rates are low. The persisting bacteriuria seems to be of little importance.
- Disk diffusion and Etest can accurately predict susceptibility to CTX and CAZ among ESBL-producing isolates, but not to TZP with the breakpoints used at the time of the study.
- Fecal carriage often persists one year after infection, and prolonged carriage is associated with *E. coli* phylogroup B2 and CTX-M-group 9.
- The host strain of ESBL-production frequently changes throughout the carriage and persisting carriage may also be linked to the transfer of plasmids carrying ESBL-genes from an infecting or colonizing strain to the normal fecal flora.
- Negative samples within the first year do not necessarily imply elimination of carriage.

This knowledge will hopefully contribute to providing better medical care of patients with ESBL-infection. It may also prove important for defining patients that require prolonged isolation in single rooms or cohorts. Thereby the spread of ESBL-producing bacteria in hospitals and long term care facilities can be limited. In the future, comparative trials are needed to further evaluate the clinical effect of antimicrobial agents against EPE-infection to make sure that we provide the best treatment available for the patients. Larger prospective studies that assess strain virulence, the effects of usage of different antibiotic classes as well as host factors such as co-morbidities on the duration of fecal carriage of ESBL are also needed. With the rapid increase in EPE-carriage these types of studies could soon be feasible also in Sweden.

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